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13. ABSTRACT ***(Maximum 200 Words)***

Receptor tyrosine kinases of the erbB family play pivotal roles in growth and differentiation and aberrant activation of these receptors is associated with human cancers. In particular, ErbB-2 dysfunction has been linked to about 30% of breast cancers with poor prognosis. Correspondingly, great efforts are being made to develop therapies that target ErbB pathways. ErbB-2 is activated by the neuregulins in heterodimers with the neuregulin receptors ErbB-3 and ErbB-4. An antagonistic neuregulin that down regulates ErbB signaling could function as an anti-tumor agent. The purpose here is to develop such a factor. In previous work, the *Drosophila* system was used to demonstrate that an antagonistic neuregulin-like factor could be made by deleting the EGF domain or by insertion of the EGF domain from a natural inhibitor. In this project, a vertebrate neuregulin-1 with an EGF domain deletion (NRGΔEGF) and a factor with the EGF domain from the inhibitor (NRG::Aos-EGF) were made. The activity of the factors is being tested in transgenic mice by examining heart defects which are characteristic of neuregulin defects. NRGΔEGF transgenic embryos show no apparent heart defects, however, data from *Drosophila* suggest the NRG::Aos-EGF will be a stronger inhibitor. Studies are underway for NRG::Aos-EGF transgenes and will be completed during a one-year no-cost extension of the project.

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Introduction

Receptor tyrosine kinases (RTKs) of the erbB family play pivotal roles in growth and differentiation during normal development. However, aberrant activation of these receptors is associated with a significant number and variety of human cancers. In particular, erbB-2 dysfunction has been linked to about 30% of breast cancers and these have a poor prognosis. Correspondingly, great efforts are being made to develop therapies that target erbB pathways. The purpose of this work is to develop a vertebrate neuregulin antagonist that has potential use as an anti-tumor agent in some breast cancers that involve erbB-2 dysfunction. The rationale for the proposed work is based on a novel finding in the fly system which shows deletion of the EGF domain, or insertion of the EGF domain from a natural inhibitor, converts the fly neuregulin, Vein, into an antagonist [1](Fig. 1). Similar modifications were made in a vertebrate neuregulin and the ability of the factors to act as inhibitors has been tested *in vitro* and *in vivo.*

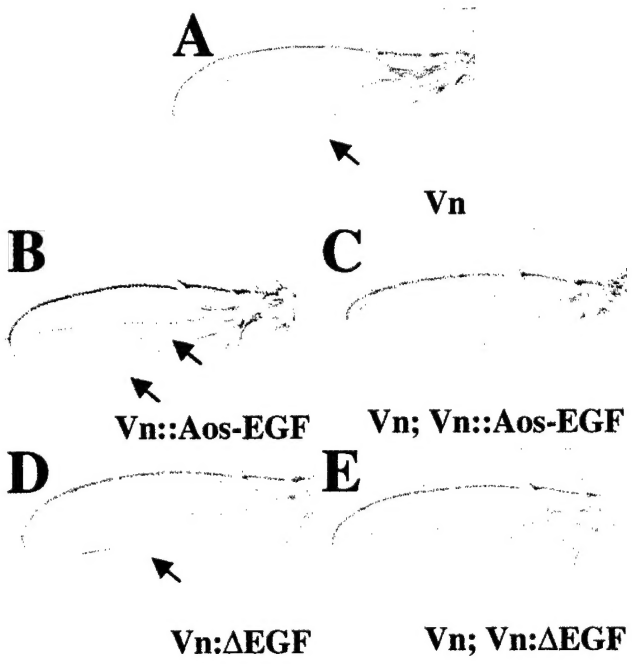

Figure 1. Vn::Aos-EGF is a more potent inhibitor than Vn:ΔEGF. A) Ectopic expression of the erbB agonist Vn causes extra veins to form indicating the EGF receptor has been activated (arrow). B) Expression of the inhibitor Vn::Aos EGF causes a loss of vein phenotype characteristic of EGF receptor inhibition (arrows) and a reduction in wing size. C) Co-expression of Vn and Vn::Aos EGF causes suppression of the extra vein phenotype, vein loss and a reduction in wing size. D) Expression of the inhibitor Vn:ΔEGF causes a loss of vein phenotype characteristic of EGF receptor inhibition (arrow) but wing size is normal. E) Co-expression of Vn and Vn:ΔEGF causes suppression of the extra vein phenotype. The extent of EGFR suppression is more pronounced following expression of Vn::Aos EGF where wing size is also affected (compare B with D and C with E).

Body

Task 1. To test the function of NRG-1: ΔEGF in cell culture.

1 a. Creation of an EGF deletion in NRG-1B

Our work in the fly system showed that deletion of the EGF domain from the fly neuregulin converted it into an erbB antagonist (Fig. 1). The goal here was to create a similar change in a vertebrate neuregulin. The NRGΔEGF construct was made as planned and we decided to also make a construct that has the EGF domain from the fly inhibitor called Argos

(Aos) (NRG::Aos-EGF). Our work in the fly system suggested this may be a more powerful inhibitor than the EGF deletion factor [1](Fig. 1).

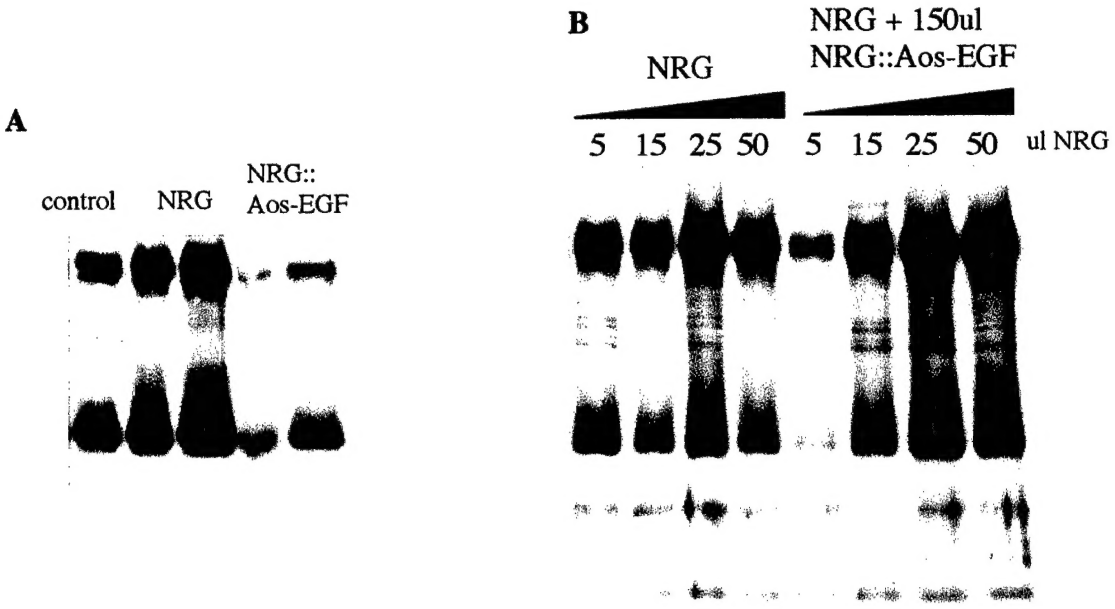

Figure 2 Inhibitory effects of NRG::Aos-EGF. ErbB4 expressing cells were exposed to control and conditioned media. The receptor was immunoprecipitated and the level of receptor phosphorylation was determined by blotting with anti-phosphotyrosine antibodies. A. The control shows the basal level of erbB4 phosphorylation (50ul of control medium was added to the cells). erbB4 phosphorylation increases upon stimulation with the agonist NRG (25ul and 50ul of NRG conditioned media were added to the cells). Addition of NRG::Aos-EGF reduced erbB4 phosphorylation below the control level (25ul and 50ul of NRG::Aos-EGF conditioned media were added to the cells). B. erbB4 expressing cells were exposed to increasing concentration of NRG (left lanes) or increasing concentrations of NRG following a pretreatment with NRG::Aos-EGF (left lanes). The level of erbB4 phosphorylation was determined. Pretreatment with NRG::Aos-EGF reduced receptor phosphorylation at the lowest concentration of NRG.

1 b. Testing effect of NRG-1: ΔEGF and NRG::Aos-EGF on NRG-1-activation of erbB receptors in mouse cells

We made large-scale preparations of DNA corresponding to the two factors (NRG-1ΔEGF and NRG-1::Aos-EGF) and sent these to our collaborator Dr. Stern at Yale medical school. Dr. Stern and colleagues transfected COS-7 cells with the DNA constructs and produced conditioned media containing the secreted factors. The activity of the factors was assayed in tissue culture cells expressing the receptor ErbB4. In this experiment the ability of the mutant factors to inhibit activation by native neuregulin was tested. Mouse cells expressing ErbB4 were treated with neuregulin or neuregulin in conjunction with a mutant factor. The receptor was immunoprecipitated and analyzed by western blotting with an anti-phosphotyrosine antibody. The level of phosphorylation is a measure of receptor activation. Most experiments were done with NRG::Aos-EGF. Unlike the parental factor NRG, NRG::Aos-EGF did not behave as an activator and could in fact reduce erbB4 signaling below baseline (the level of signal when control medium was added) (Fig. 2A). Furthermore, NRG::Aos-EGF was able to reduce

activation of the receptor by NRG (Fig. 2B). These results were consistent with the factor acting as an inhibitor and we therefore began testing in cancer cells.

1 c. Testing effect of NRG-1: ΔEGF on human breast cancer cell lines

In these experiments we tested the ability of the mutant neuregulins to block signaling through erbB receptors in human breast cancer cells. The cancer cell lines tested in these experiments were MDA-MB-453, MDA-MB-468, MCF7, and MDA-MB-175-VII. The control neuregulin (pHM1-NRG) and the mutant factors (pHM1-NRGΔEGF, pHM1-NRG::Aos-EGF) were produced by transfecting COS-7 cells. Conditioned media were collected and concentrated to 1/10 volume using Ultrafree centrifuge columns (Millipore). The concentrated media was then applied to the cancer cells after overnight starvation in 1% FBS. Cell lysis and blotting were then done. For the MDA-MB-175-VII cells, immunoprecipitation with anti-ErbB3 followed by blotting with anti-phosphotyrosine antibodies was performed. For the MDA-MB-453, MDA-MB-468 and MCF7 lines, immunoprecipitation with anti-MAPK followed by blotting with anti-phospho-MAPK was done. Results from these blots did not show any difference between the control and cells treated with the potential inhibitors.

In order to check that the inhibitors were being efficiently produced we wished to examine protein levels. The proteins are Myc-tagged but the tag is on the C-terminal part of the proteins, which is cleaved during secretion, and is likely degraded and therefore not useful for detection of secreted protein. Hence, an HA tag was put on the N-terminal portion via PCR. A small portion of the N-terminal region is also cleaved during the secretion process, so the HA tag was placed inside this cleavage point. Repeated attempts were made to detect the HA-tagged proteins. Conditioned media, concentrated conditioned media, and whole-cell lysates were assayed. Positive controls confirmed the transfections were successful and a non-secreted positive control for the HA tag demonstrated the antibodies were working. We will continue to work on these technical issues during a one-year extension of this project

Task 2. To generate and analyze the phenotypes of transgenic mice that express NRG-1: ΔEGF in heart and breast.

2 a. Creation of NRG-1: ΔEGF transgene for expression in early embryos

We proposed to test the ability of the mutant neuregulins to function as dominant negative proteins that mimic loss of function heart phenotypes seen in neuregulin knockout mice [3]. To do this we made a construct suitable for generating transgenic mice that express the gene in the heart under control of the α-myosin heavy chain promoter. We also made an additional construct with the EGF domain from Argos (NRG::Aos-EGF) because this factor appears to be a more potent inhibitor in flies (Fig 1). Furthermore Vinos and Freeman (2000)[2] showed that some mammalian cell types can indeed be inhibited by *Drosophila* Argos suggesting that an interaction between the Argos EGF domain and a vertebrate receptor is possible. Thus on balance it seemed prudent to make this additional transgene.

2 b. Generation and phenotypic analysis of NRG-1: ΔEGF transgenic mice

Neuregulin knockout mice die in mid embryogenesis of heart defects [3]. Thus we reasoned that the quickest way to test the efficacy of the inhibitors, which should act as dominant factors, would be to test whether they induce heart defects. To direct expression of the transgenes to the heart we used the α-myosin heavy chain promoter. In theory if the transgene were functioning as expected no transgenic mice should have been produced. Thus the hoped for result was negative; that no lines be produced. Nevertheless, it was essential that we conducted this experiment because the factors may not function in mice as they do in flies. If this were the case we would recover normal numbers of healthy transgenic mice. It turned out that the results were not 'cut and dried'- we got some mice but at lower than expected frequencies.

One founder mouse with the NRG:ΔEGF construct was created, after several injection attempts that produced a total of only nine mice. This low recovery was in keeping with our hypothesis that the transgene would act as a dominant negative inhibitor of neuregulin and be embryonic lethal. However, the Children's Hospital transgenic facility suffered technical problems at the time our mice were generated. (We therefore decided to use an alternative facility on campus for subsequent injections, see below.)

F1 and F2 litters have been produced from the NRG:ΔEGF founder and Southern blotting shows copy number estimates for the transgene are between 1 and 10. To determine whether this line produces viable progeny because the transgene is not efficiently expressed in the heart we performed reverse transcription of RNA from tissue samples followed by PCR to amplify transgene specific sequences (RT-PCR). Initially expression in the heart was not detected in all animals (as stated in the midterm report). However, assays with subsequent litters showed expression of the transgene in all transgenic individuals (multiple litters have been tested, two shown in Figs. 3 and 4). We are currently analyzing protein levels in the heart.

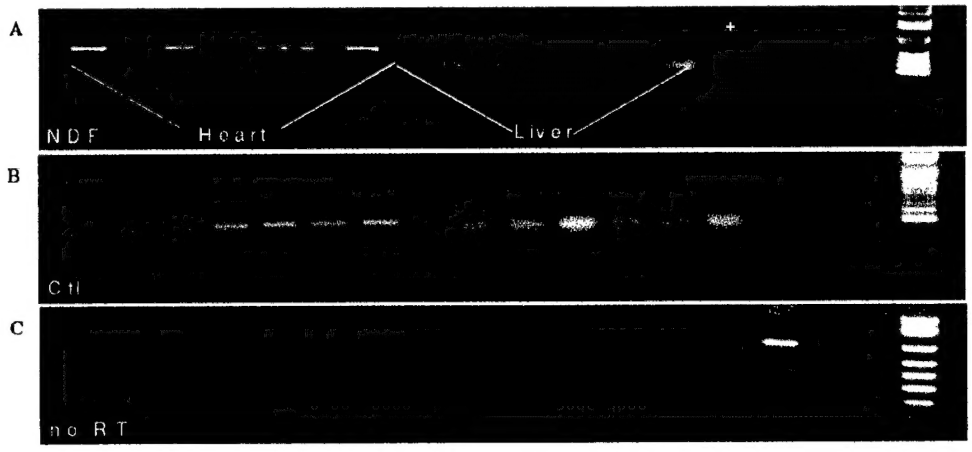

Figure 3. RT-PCR with an F1 NRG:ΔEGF litter. A. Transgene-specific primers. Only the transgenic mice (1,2,3,5,6,7) showed expression in the heart. B. Alpha-myosin heavy chain primers, positive control. C. No-RT control.

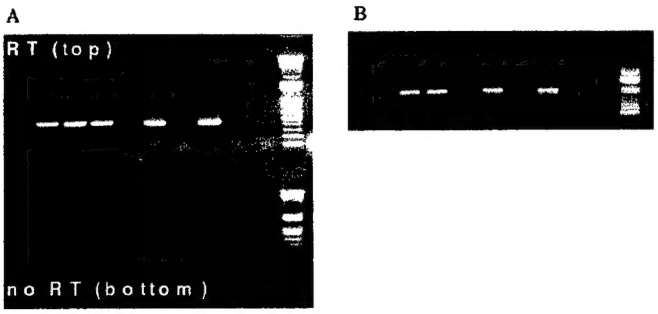

Figure 4. RT-PCR with an F2 NRG:ΔEGF litter. A. Transgene-specific primers, showing expression in hearts of the transgenic mice (no expression seen in the liver samples, data not shown). B. PCR screen of the F2 litter to compare with RT results, showing that only transgenic animals (1-3, 5 and 7) showed expression.

We are also testing NRG::Aos-EGF, which appears to be a stronger inhibitor in flies, and thus switched our efforts into analyzing transgenic mice with this construct. To test the function of NRG::Aos-EGF transgene we used the Keck transgenic mouse facility at Ohio State University. This transgenic mouse facility guarantees the production of at least 30 candidate mice of, which typically about half are transgenic. The NRG::Aos EGF construct yielded only 1 transgenic founder out of 22 mice and this mouse failed to transmit the transgene. This failure to generate a transgenic line is in keeping with our original hypothesis that the transgene is dominant lethal, but technical problems could also explain the result.

We thus tested directly for embryonic lethality caused by the NRG::Aos-EGF transgene in a transient transgenic experiment. Injections were performed and the embryos were harvested at day E11. A total of 23 embryos were tested and 7 of these were found to be transgenic. No gross heart defects in the embryos were observed by our colleague Dr. Michael Weinstein. Dr. Weinstein is an experienced mouse embryologist. Two transgenic embryos and two non-transgenic embryos were sectioned and their hearts examined in detail. No defects were detected (Figs. 5 and 6). In neuregulin knock out mice, trabeculation defects are seen by day E11 [3]. *In situ* hybridization showed that the transgene was expressed in the heart, albeit at lower levels than the alpha-myosin heavy chain control (Fig. 7). Despite transgene expression, trabeculation appeared normal. These results are consistent with the idea that the transgene is not acting as a neuregulin inhibitor and that the failure to recover stable lines was due to technical problems in generating the transgenic mice. Still, we cannot exclude the possibility that the transgene is causing a lethal phenotype (hence the failure to recover stable lines) and that a heart defect develops later than day E11. It would not be surprising to find that misexpression of an inhibitor fails to mimic the null phenotype exactly.

Figure 5. Cross-section of a wild-type day 11 embryo heart, showing normal trabeculae.

Figure 6. Cross-section of a NRG::Aos-EGF transgenic day E11 embryo. The embryo has normal trabeculation similar to its wild-type littermate (Figure 5).

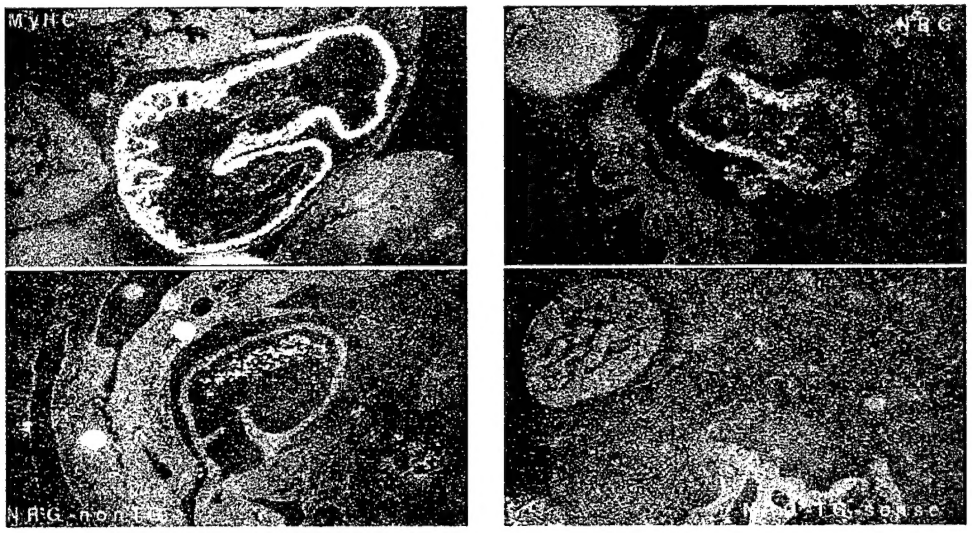

Figure 7. *In situ* hybridization in day E11 embryos,. Expression of NRG::Aos-EGF is seen in the heart (anti-sense probe, top right; sense probe, bottom right). Transgene expression was driven with the α-myosin heavy chain promoter and strong expression of α-myosin heavy chain is seen in the heart (top left). Non transgenic mice do not show expression demonstrating the probe is specific to the transgene (bottom left).

To resolve the issue of whether later defects could be caused by NRG::Aos-EGF expression we have had an additional injection to create NRG::Aos EGF transgenic mice conducted by Jon Neumann at the University of Cincinnati. This is an outstanding facility well known for the production of transgenic mice. Thirty-five mice were produced and 2 of these were found to be transgenic. They have been transferred to Ohio State University and are currently in a quarantine facility where they have been mated and the first litters will shortly be forthcoming.

We believe these mice will be key to resolving the question as to whether the transgene acts as an inhibitor. If the mice express and transmit the gene without detrimental effects we can conclude that the inhibitor is unlikely to be useful in any subsequent work. However if the mice either fail to transmit the transgene (they are mosaic) or express the gene at only low levels this would support pursuing the project further.

We have been granted a one year no-cost extension for this work and will use this time to complete the analysis of the transgenic mice in hand. If the results support the idea the factor behaves as an inhibitor we will perform a detailed analysis of transient transgenic mice expressing NRG::Aos-EGF. We will harvest embryos at multiple time points (E11-E15) and examine them for gross heart defects and for more subtle defects in sections. Our colleague Dr. Weinstein has agreed to work with us on this project as he has a great deal of experience with analyzing embryonic lethal mutations in mice.

The remaining tasks (2 c&d and 3) described in our original plan are currently on hold. Tasks 2 c&d were to create MMTV NRG:ΔEGF and MMTV NRG::Aos EGF transgenic mice. There is no sound rationale for initiating these studies at the present time. However, if we find clear evidence that NRG::Aos-EGF behaves as an inhibitor in the heart we will pursue analysis of NRG::AOS EGF in the breast and test the efficacy of the factors in suppressing tumor development in a mouse breast cancer model (Task 3).

Key Research Accomplishments

- Demonstration that expression of a NRGΔEGF transgene in the heart does not cause lethality in mice
- Demonstration that transgenic mice expressing NRG::Aos EGF do not have heart defects on day E11
- Generation of two NRG::Aos EGF transgenic mice

Reportable Outcomes

- We plan to publish this work either as a stand-alone study or in conjunction with work in the *Drosophila* system. How we proceed will be determined by the outcome of the last transgenic mouse experiment, which will be completed during a one-year no-cost extension.
- We have developed a NRGΔEGF transgenic line that is apparently normal and are characterizing two transgenic mice harboring a NRG::Aos EGF transgene.

Conclusions

Work with erbB signaling in *Drosophila* showed that a stimulatory factor, the fly neuregulin called Vein, could be converted into an inhibitor by either deleting the EGF domain or inserting the EFG domain from a natural inhibitor called Argos [1] (Fig. 1). This prompted the question as to whether such mutant factors would function similarly in vertebrates. To investigate this possibility we have generated transgenic mice with copies of altered neuregulin (NRG). Studies with transgenic mice carrying the deletion construct, NRGΔEGF, showed that expression of transgene in the heart did not cause a detectable phenotype. (In a last set of experiments on this mouse line we are checking protein expression in the heart.) Work with *Drosophila* showed that the factor containing the EGF domain from Argos was a stronger inhibitor than the deletion form (Fig. 1). Therefore we are also examining the activity of a NRG::Aos EGF transgene in mice. We have studied this gene in transient transgenic experiments. Embryos injected with the NRG::Aos-EGF transgene were harvested and examined for heart defects at day E11. $NRG^{-/-}$ mice show trabeculation defects by this stage [3], but, no such defects were seen following expression of NRG-Aos EGF in the heart. However, misexpression of an inhibitor may not faithfully recapitulate the null phenotype and the embryos may show defects later in development. We will test this possibility in two further experiments. First we have generated two mice with the NRG::Aos-EGF transgene and are breeding these to see if we can establish stable lines that express the protein in the heart. If this is found to be the case we will conclude NRG::Aos EGF fails to inhibit NRG function in the heart. If we cannot establish lines we will extend the transient transgenic studies to examine heart defects at later stages.

We have been granted a one-year no-cost extension for this project and this time will be used to complete these final experiments. Without these results we are not in a position to include a "so what section".

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